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Commentary

Transforming Growth Factor-β_1 Knockout Mice

A Mutation in One Cytokine Gene Causes a Dramatic Inflammatory Disease

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Knockout mice have become an excellent tool to create mouse models for genetic diseases and to elucidate the essential functions of important regulatory molecules (products from growth factor genes, oncogenes, and developmental genes). Knockout mice are generated by gene targeting in embryonic stem (ES) cells. These mutated ES cells are injected into the recipient embryos to generate chimeric mice that contain tissues (including germ cells) derived from the mutated ES cells and pass the trait on to their offspring (see Figure 1 and refs. 4 to 7).

Gene Knockout Astrology: The Prediction of a Phenotype

Predicting a phenotype for a particular gene knockout mouse can be difficult, in particular when no known disease is associated with the mutated gene in question. A knockout mouse harboring a null mutation develops a "subtraction" phenotype, ie, the phenotype is caused by functional losses that cannot be compensated for by other proteins. The degree of functional redundancy determines the extent of the phenotype. An extreme example of this is the mutation in the hypoxanthine phosphoribosyl transferase gene that did not cause any clinical symptoms (complete redundancy), in sharp contrast to the Lesch-Nyhan syndrome seen in patients with hypoxanthine phosphoribosyl transferase deficiency.[8,9] Detection of the phenotype may be problematic if it is subtle and detailed analysis may be required to reveal important defects.[10] Additional difficulties arise when an embryonic lethal mutation is created. The loss-of-function mutation will reveal the role of the gene during development, but any possible postnatal roles cannot be analyzed.

Despite these limitations, the knockout technology is a very powerful tool to generate models of human disease. Knockout mice can similarly be used to unravel molecular mechanisms in many biological systems during both embryonic and postnatal development. Table 1 describes deficiencies in lymphohematopoietic cells of knockout mice that have been published to date. Many functional defects have been detected in the immune system of these mice. Genes whose deletion results in a detectable phenotype include both genes that act mainly within the immune system and those that have a broader function. Tumor suppressor genes, oncogenes, and growth factor genes have been popular targets in order to establish their role in cell growth and differentiation. In this paper we will discuss one of these, the transforming growth factor-β_1 (TGF-β_1) gene, its inactivation, and null phenotype.

TGF-β

TGF-β is a multifunctional cytokine with diverse biological actions.[34–39] These actions influence a wide range of cellular, physiological, and immunological processes. TGF-β is known to regulate cell prolifer-

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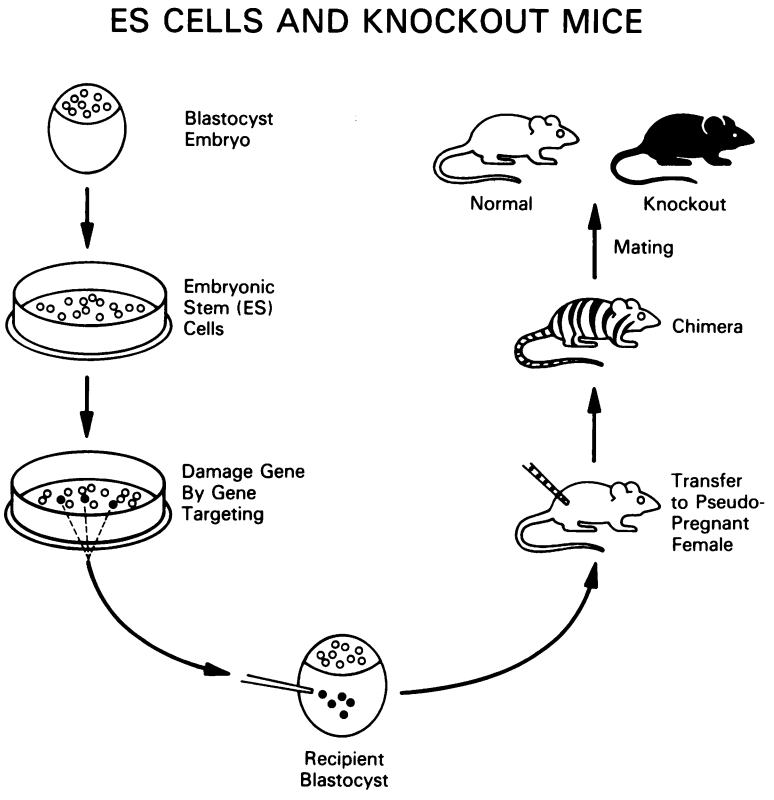

Figure 1. *ES cells are generated from the inner cell mass of a 3.5-day-old blastocyst embryo.[1,2] They are grown* in vitro *like other cell lines and can therefore be manipulated genetically as other cell lines. A DNA construct containing a mutated mouse gene is transferred into the ES cells and clones that are likely to have undergone gene targeting (growth in selective medium) are characterized by molecular techniques to confirm the gene targeting (or knockout) event.[3] The mutated gene construct has replaced the normal endogenous gene, most often resulting in a null allele. The mutated ES cells are injected into the 3.5-day-old blastocysts and transferred to the uterus of pseudopregnant foster mothers to generate chimeras as shown. Germline chimerism is proved by mating the chimera. When the mice are born that have the same coat color as the strain that generated the ES cells, germline chimerism is proved and a transgenic line containing the knockout gene has been successfully generated.[4–7]*

ation and differentiation, and its suppressive action on epithelial cells implicates a role in carcinogenesis. Its profound effects on extracellular matrix formation play a crucial role in cellular morphogenesis, bone remodeling and development, fibrotic disease processes, and wound healing. In the last few years it has become apparent that TGF-β also plays an important role in immune functions. TGF-β_1 is a potent immunosuppressor. During inflammation its role is complicated because it appears to have a dual role (proinflammatory at first and later acts as an immunosuppressor) as reviewed by Wahl.[35] In recent years many laboratories have reported potential clinical applications for TGF-β in wound healing in soft tissues, bones, retina, brain and heart, and in autoimmune disease and in protection from ischemic cell death in brain and heart.[40] Dysregulated expression of TGF-β is also associated with fibrotic and proliferative diseases.

Three distinct TGF-β genes have been identified in mammals: TGF-β_1, TGF-β_2, and TGF-β_3. Although these three genes have been highly conserved throughout evolution suggesting specific roles for each of them, these isoforms are often co-expressed and co-localized and exhibit overlapping biological actions. TGF-β_1 is a prototype for the TGF-β family with essentially similar but specific roles in different target tissues and cells. In view of this complex expression pattern and profound role in mammals, it is important to create mouse models lacking each of these isoforms to begin to define their roles for each of them.

The TGF-β_1 Null Mouse

We have successfully targeted the entire TGF-β_1 gene including its precursor region (coding for the latency-activating protein (LAP)) in mouse embryonic stem cells and used these mutant cell lines to create transgenic TGF-β_1 null mutant (TGF-β_1 –/–) mice.[21] The TGF-β_1 null mouse has also been successfully created by inactivating the TGF-β_1 gene in exon 6.[22] The latter strategy made use of a fusion protein that forms most of the TGF-β_1 coding sequences (including the LAP region) together with the neomycin resistance gene sequences.

TGF-β_1(–/–) mice do not produce detectable TGF-β_1 messenger RNA or protein indicating a definite TGF-β_1 null mutation. A considerable intrauterine lethality is associated with this mutation because less than half of the expected number of TGF-β_1(–/–) mice are born. These mice are born healthy and remain clinically normal for 2 weeks. By

Table 1. *Knockout Mice with Deficiencies in Lymphohematopoietic Cells*

Gene	Phenotype	Authors
Tumor suppressor genes		
Rb	Neuronal cell death and defective erythropoiesis	Jacks et al[11] Lee et al[12]
Oncogenes		
c-*abl*	B and T cell lymphopenia, thymic and splenic atrophy: die at 1–2 weeks of age	Schwartzberg et al[13] Tybulewicz et al[14]
c-*fos*	Osteoporosis, altered hematopoiesis	Johnson et al[15] Wang et al[16]
c-*myb*	Defects in fetal erythropoiesis; die at 15 days of gestation	Mucenski et al[17]
c-*src*	Osteopetrosis	Soriano et al[18]
Growth factor genes		
IL-2	Dramatic changes in immunoglobulin isotypes; abnormal T cell response *in vitro*	Schorle et al[19]
IL-4	Strong reduction of IgG_1 and IgE	Kühn et al[20]
TGF-β_1	Multifocal inflammation; die at 3–4 weeks of age	Kulkarni et al[21] Shull et al[22]
Immune system genes		
β_2-microglobulin	Absence of class I antigens; lack $CD4^-8^+$ cytolytic T cells	Zijlstra et al[23] Koller et al[24]
MHC II $A^b\beta$	Depletion of mature $CD4^+$ cells; deficiency of CMI	Grusby et al[25]
μ-Chain	B cell deficiency; loss of heavy chain allelic exclusion	Kitamura et al[26] Kitamura and Rajewsky[27]
RAG-1	Loss of mature B and T lymphocytes	Mombaerts et al[28]
RAG-2	Lack of mature lymphocytes (SCID phenotype)	Shinkai et al[29]
TAP-1	Deficient antigen presentation; surface class I molecules and $CD4^-8^+$ T cells	Kaer et al[30]
5′Sequence of Sγ 1 switch region	Shutdown of class switch recombination, selective agammaglobulinemia	Jung, Rajewsky, and Radbruch[31]
Transcription factor gene		
GATA-1	Block of erythroid differentiation	Pevny et al[32]
Human disease gene		
Glucocerebrocidase	Gaucher's disease, type II; die on day 1 after birth	Tybulewicz et al[33]

the end of the second week TGF-β_1(−/−) mice develop a rapid wasting syndrome and weigh only 50% of their normal littermates at the time of their death at 3 to 5 weeks of age. These mutant mice have an excessive inflammatory response and develop a multifocal inflammatory disease resulting in cardiopulmonary complications that are ultimately lethal. Histopathological analysis of these mice revealed massive infiltration of lymphocytes and macrophages in many organs, but primarily in heart and lungs (Figure 2). In the heart, inflammatory lesions included endocarditis and myocarditis in all TGF-β_1(−/−) mice analyzed. In some cases pericarditis, myocarditis, and thrombosis were also observed. In the lungs, the inflammatory reaction caused vasculitis (phlebitis) and interstitial pneumonia. In the spleen and the lymph nodes, inflammatory lesions included proliferation of immunoblasts and lymphoblasts in B- and T-cell zones. Inflammatory lesions were also seen in pancreas, salivary glands, colon, and stomach of some of the TGF-β_1(−/−) mice. Interestingly, the phenotypes of the two TGF-β_1 null animal models[21,22] are very similar; however, there are some differences. Shull et al[22] have reported significant inflammatory lesions in the liver, stomach, and striated muscle of the TGF-β_1 null mice. Our mice have much less significant inflammation in stomach or liver and none in muscle. This could be caused by differences in the mouse strain (CF1 *versus* C57BL/6J in our case) used in generating the TGF-β_1 null mouse. Alternatively, the presence of the fusion protein containing the LAP protein sequences may determine some of the phenotypic differences, although this may not be likely. It would be of interest to compare the detailed phenotypes of these two TGF-β_1 knockout mice to unravel potential biological roles of the LAP region.

How this massive inflammatory response is triggered is unclear at present. Some of the inflammatory lesions such as vasculitis, interstitial pneumonia, and sialoadenitis found in these mice are similar to those caused by certain murine pathogens. Graft-*versus*-host reaction in mice also produces histopathological lesions similar to those found in TGF-β_1(−/−) mice; however, the pattern of these lesions in graft-*versus*-host reaction differs

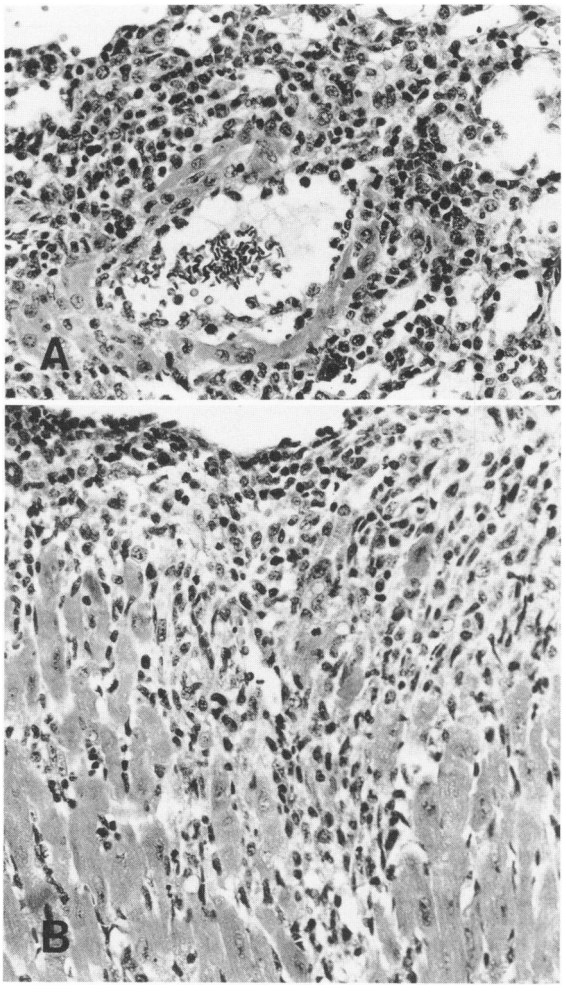

Figure 2. *Histopathology of pulmonary vein and heart of the TGF-β_1(−/−) mouse.* A: *Pulmonary vein showing phlebitis. Lymphocytes and macrophages are seen in and adjacent to the vein. Note cardiac muscle in wall of vein. H & E. ×250.* B: *Endocarditis showing macrophages and lymphocytes infiltrating myocardium. H & E. ×250.*

from the one seen in TGF-β_1(−/−) mice. MHC class I and II expression was found to be significantly elevated in the young TGF-β_1(−/−) mice preceding the development of the inflammatory disease (A. Geiser and J. Letterio, unpublished data). These findings suggest that inflammatory lesions in the TGF-β_1(−/−) mice may be autoimmune in nature because TGF-β has been shown to delay the onset and reduce the severity of experimental autoimmune disease in animal models of multiple sclerosis[41] and rheumatoid arthritis.[42] Shull et al[22] have found increased peripheral white blood cell and neutrophil counts in their TGF-β_1 mutant mice. This was accompanied with increased levels of interferon-γ, tumor necrosis factor-α and macrophage inflammatory protein-1 messenger RNA in liver and lungs. In our studies, TGF-β_1(−/−) mice also had elevated levels of interferon-γ and their splenic cells showed aberrant proliferative responses to mitogens (S. Wahl et al, unpublished data). Elevated levels of inflammatory cytokines suggest an uncontrolled "cytokine storm"[43] because of the lack of TGF-β_1 in these mice. Detailed analysis of the TGF-β_1(−/−) mice phenotype will provide us with more insight into molecular mechanisms of TGF-β_1 in the immune processes.

In normal mice, large amounts of TGF-β are found in bone and cartilage matrix and high levels are synthesized in growth plate chondrocytes during longitudinal bone growth. Radiographs of the skeleton demonstrated grossly normal bone morphology and skeletal architecture in the TGF-β_1-(−/−) mice. However, histopathological analysis revealed thinner (70% of normal controls) proximal tibial growth plates in the symptomatic 3-week-old knockout mice (T. Ballock et al, unpublished data) and the chondrocyte columns in these plates appeared irregular. Similarly, the histological appearance of testes was abnormal in the symptomatic knockout mice suggestive of maturation arrest (K. Boekelheide et al, unpublished data). It is not clear at this stage whether systemic illness associated with excessive inflammation in these mice is a contributive and/or a causative factor for the abnormalities seen in growth plates and testes or whether these are developmental defects.

TGF-β has been shown to be expressed in the epidermis and is identified as a growth inhibitor of mouse keratinocytes *in vitro*. TGF-β_1(−/−) mice have a three- to fivefold increase in epidermal labeling index compared with normal mice. This increase in keratinocyte proliferation suggests that TGF-β_1 may be an *in vivo* negative regulator of skin growth. Lack of TGF-β_1 may also contribute to skin carcinogenesis (A. Glick et al, unpublished data). Because high risk papillomas do not express TGF-β and are associated with hyperproliferation, TGF-β_1 loss in the knockout mouse will be a valuable marker to study malignant progression.

Future Directions

The TGF-β_1(−/−) phenotype was not totally unexpected because it is well known that TGF-β_1 is a potent anti-inflammatory molecule. However, because TGF-β_1 also has powerful growth regulatory functions with multiple roles in many biological systems as described earlier, we anticipate detection

of additional abnormalities in these mice. The serious systemic illness that these mice develop may mask several other developmental and cellular defects.

Added exogenously, the three isoforms of TGF-β have essentially similar biological actions; however, it is clear that expression of each of the three isoforms is regulated in an unique tissue-specific pattern *in vivo*. The TGF-β_1(−/−) mouse will be an invaluable model to unravel the functional redundancy in the three isoforms and to identify specific biological roles of TGF-β_1. Study of the anti-inflammatory activities of TGF-β provides an opportunity to delineate its role in: 1) growth inhibition of activated T lymphocytes by its interaction with Rb gene phosphorylation, inhibition of IL-1 receptor expression, and enhanced expression of IL-1 receptor antagonist; 2) regulation of $CD8^+$ and $CD4^+$ lymphocytes and synthesis of potentially inhibitory cytokines such as IL-4, IL-5, and IL-10; 3) inhibition of uncommitted hematopoietic progenitors; 4) regulation of cytolytic functions of T, LAK, and NK cells; 5) blocking of B cell-proliferative response; 6) switching of IgM- or IgD-bearing B cells to those bearing IgA isotypes, and regulation of expression of both classes of major histocompatibility molecules. In the absence of TGF-β_1, one would expect to see down-regulation of proinflammatory actions of this isoform. This mouse model will help to evaluate the functions of TGF-β_1 in the recruitment of inflammatory cells (regulated through enhanced integrin expression, enhanced monocyte-matrix adhesion, and increase in matrix-specific collagenase secretion and chemo-taxis) and activation of mononuclear cells (result of triggering the cascade of proinflammatory cytokines and differentiation of T and B cells), and will provide insight into the role of TGF-β_1 in disorders associated with inflammation, autoimmunity, graft-*versus*-host reaction, and transplant rejection.

TGF-β has been implicated in embryonic development including implantation of blastocysts. Because more than half of the knockout embryos do not come to term, the study of the mechanism of intrauterine lethality will shed new light on the role of TGF-β_1 in embryogenesis. Moreover, the primary and transformed cell lines derived from the TGF-β_1-(−/−) mice and the littermate controls will provide ideal systems for *in vitro* investigations, free of the inflammatory effects that complicate analysis *in vivo*.

Because TGF-β plays an important role in cell proliferation and differentiation, cell adhesion and migration, extracellular matrix protein expression, and angiogenesis, it was surprising that no obvious major developmental defects were observed in the TGF-β_1(−/−) animals. This may be caused by compensatory actions of other growth factors that can replace the TGF-β_1 loss (functional redundancy) or by masking of subtle developmental defects by the systemic illness. The answers to these questions may not be clear until an effective treatment for the inflammatory disease with cytokines or cytokine antagonists is developed.

The mice can also be used to investigate the role of TGF-β_1 in wound healing, bone remodeling, angiogenesis, and other physiological processes. Carcinogenesis, tissue damage caused by ischemia, and other pathological processes can also be investigated using these mice, and this includes the evaluation of therapeutic agents for these conditions.

In summary, the TGF-β_1 knockout mouse has a multifocal inflammatory disease caused by the abrogation of the anti-inflammatory and immunosuppressive effects of TGF-β_1. This "subtraction" phenotype indicates absence of complete redundancy in the TGF-β family of growth factors and opens a new dimension for investigating the multiple roles of TGF-β *in vivo* and *in vitro*.

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